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MYCOFLORA AND AFLATOXIN/FUMONISIN PRODUCTION BY FUNGAL ISOLATES FROM FRESHLY HARVESTED CORN HYBRIDS

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ABSTRACT

The mycoflora of 3 hybrids of freshly harvested corn grains collected from three regions of the state of São Paulo, Brazil (Assis, Capão Bonito and Ribeirão Preto) was investigated. A total of 66 samples were analyzed focusing on the influence of abiotic factors (moisture content, water activity, temperature and rainfall) on both the prevalence of *Aspergillus flavus* and *Fusarium moniliforme,* and the ability of these genera isolates to produce aflatoxins and fumonisins, respectively. In the three surveyed regions, the fungal population comprised mainly *Fusarium* spp., *Penicillium* spp., *Aspergillus* spp. and 2 others filamentous fungal genera, which were isolated from corn kernels showing water activity of 0.30 to 0.99 and moisture content of 5.0% to 20.2%. Among the genera *Fusarium* and *Aspergillus*, the most frequent species were *F. moniliforme* and *A. flavus*, respectively. Concerning the toxigenic potential of *F. moniliforme*, all isolated strains (40) produced fumonisins at 20 μg/g to 2168 μg/g (FB_1) and/or 10 μg/g to 380 μg/g (FB_2). From the 10 *A. flavus* isolates, 6 strains (60.0%) produced aflatoxins at 615 μg/kg to 30.750 μg/kg (AFB_1) and/or 11 μg/kg to 22 μg/kg (AFB_2).

Key words: aflatoxins, *Aspergillus flavus*, fumonisins, *Fusarium moniliforme,* corn

INTRODUCTION

The corn in Brazil has an important role in both human and animal nutrition, and has been ranked as the third world producer following the USA and China. Recently, the Brazilian annual corn yield reached 32 tons (32), of which 41% was used for poultry and swine consumption, where approximately 24.7% is directly used in farms, produced significantly by small producers (26).

The mean productivity of 2.747 kg corn/ha recorded in the state of São Paulo in 1990-94 reflects a poor local technology (31). The corn fields in this state correspond to 7% of cultivated land and cover a mean area of 13.28 ha (10); 61.93% of the farmers receive technical assistance, 44.14% use soil analysis, and 46.23% use hybrid grains (10).

Grain spoilage reduces the nutritional value of cereals, resulting in world losses of foodstuff in 5% (14). Improved storage conditions can rise 10-20% in the supply of foodstuffs (8), as in Brazil, losses of 10% (26) to 25% (6) occurs during trading.

Fungi are worldwide microorganism, although tropical climates favor the growth of toxigenic species on agricultural products, with consequent risk of mycotoxin contamination (12). Therefore, as local agricultural practices and storage characteristics can be expected to create optimal growth conditions for specific toxigenic fungi, the need for regional investigations on these parameters cannot be neglected. The aim of this work was to evaluate the contamination risk of corn cultivated in the state of São Paulo, Brazil, by studying the mycoflora of three hybrids in three different regions, focusing on the influence of abiotic factors (moisture content, water activity, temperature and rainfall) on the prevalence of fungal species as well as the ability of *Aspergillus flavus* and *Fusarium moniliforme* isolates to produce aflatoxins and fumonisins, respectively.

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MATERIALS AND METHODS

Grain samples

Three corn cultivars (hybrids BR-201, C-901 and CX-322) of 1995 crop were selected according the harvest period required from seeding to harvest. The hybrids were cultivated in three regions of the state of São Paulo: Assis (22°40' latitude, 50°28' longitude), Capão Bonito (24°02' latitude, 48°22' longitude) and Ribeirão Preto (22°11' latitude, 47°48' longitude), Brazil. Sixty six samples were analyzed, 30 of which were from Assis, 18 from Capão Bonito and 18 from Ribeirão Preto.

Moisture content and water activity

The moisture content of the corn grains was determined in the storage "*in loco*" immediately after sampling, using a "Brow Duvel" moisture meter (Model CA 25II, Gehaka Co.). Water activity was determined by automated analysis using AQUALAB CX-2 (Decagon Devices Inc.). Each sample was measured five times.

Recovery, identification and enumeration of the mycoflora (5)

Ten grams of sampled corn was ground and mixed with 90 ml of sterile distilled water, followed by ten fold serial dilutions up to 10^{-4}. Duplicate 1 ml volumes of each dilution were added to Petri dishes containing 10 to 15 ml of Potato Dextrose Agar (PDA); the plates were incubated at 25°C for 5 days and observed daily. Plates that contained 15 to 150 CFU were used for counting and the results were expressed as CFU per gram of sample. The fungal colonies recovered were identified according to methods recommended for each genus (2,22,28).

Toxicity of *F. moniliforme* strains

One ml spore suspensions of *F. moniliforme* strains cultured in Sabouraud Dextrose Agar (SDA) at 25°C were inoculated into Erlenmeyer flasks containing 50 g of sterilized rice. The Erlenmeyer flasks were incubated for 15 days at 25°C, followed by another period of 15 days at 15°C. The rice samples were then tested for fumonisins according to the method of ORSI *et al.* (25). Briefly, 10 grams of rice cultures were added to 50 ml of acetonitrile/water (1:1) and stirred for 30 minutes. The extract was filtered, 2 ml of the filtrate was added to 5 ml of water and the mixture was applied to a preconditioned Sep-pak C-18 cartridge (Waters, Division of Millipore, Milfort MA). The cartridge was washed with 2 ml of acetonitrile/water (20:80) and the toxin eluted with 2 ml of acetonitrile/water (70:30). The final extract was collected in Eppendorf tubes and stored at –20°C until use.

Two hundred microliters of the final extract were derivatized with 50 µl of o-phthaldialdehyde (OPA) (40 mg of OPA dissolved in 1 ml of methanol and diluted in 5 ml of 0.1 M sodium tetraborate containing 50 µl of mercaptoethanol). The reaction product was analyzed by a reverse-phase isocratic HPLC system consisting of a Shimadzu SCL-6B pump, an RF55 fluorescent detector (Shimadzu; excitation and emission wavelength of 355 and 440, respectively) and a 150 x 4.6 mm C18 column (50DS-20, Phenomenex). The eluent was methanol/sodium acetate buffer (77:23) pH 3.6. Calibration of the apparatus was made using solutions of standard fumonisins (Sigma) at the concentrations of 0.0125, 0.025 and 0.05 µg/ml for FB_1 and 0.005, 0.01 and 0.02 µg/ml for FB_2. A recovery test was conducted in quadruplicates at levels that ranged from 4 to 24 ng of FB_1 and 8 to 56 ng of FB_2 per g of ground corn. The recoveries of FB_1 and FB_2 were 88% and 94%, respectively. The detection limit was 50 ng/g for both FB_1 and FB_2,with a minimum detectable concentration of 10 ng/g.

Toxicity of *A. flavus* strains

A small fragment of *A. flavus* colony in SDA at 25°C was inoculated on the center of a Petri dish with Coconut Agar (21). Incubation was carried out at 25°C for 10 days, and the cultures were assayed for aflatoxins as describe by Lin and Dianese (21). The Coconut Agar cultures were extracted with chloroform (30 ml chloroform per 10g culture) by shaking for 30 minutes. The content was filtered through a Whatman #1 filter paper and evaporated to dryness. The suspended extracts were quantified by thin-layer chromatography (TLC) using standard aflatoxins (Sigma) (1).

Climatic data

Temperature (°C) and rainfall (mm) were recorded as monthly averages by the Agronomic Institutes of Assis, Capão Bonito and Ribeirão Preto.

Statistical analysis (9,30)

The statistical analysis of the data was performed in two stages, using the Statistical Software SAS (SAS Institute, 1985):

1) In the preliminary analysis the variables were selected accounting the growth of *Fusarium* spp., which can also indicate differences between hybrids per region. To determine the multiple regression model, simple correlation and partial correlation analyses were performed, so as to describe the nature of the relationship between the dependent variable (*Fusarium* spp growth) and the independent variables (water activity, moisture content, mean temperature and rainfall). Next, the multiple linear regression model was determined taking into account all the variables of the experiment. Variables were then selected by the Stepwise Method and a residual analysis of the chosen model was performed. The analysis of parallel lines was applied in order to test for possible differences between hybrids per region.

2) In a second stage, a comparison of means was performed to analyze, for each hybrid, the effect of regions on *Fusarium* spp. growth.

RESULTS AND DISCUSSION

The analysis of 66 samples of freshly harvested corn grains (hybrids BR-201, C-901 and CX-322) collected in three regions of the state of São Paulo revealed the following compositions of fungal microbiota: Region of Assis, - *Fusarium* spp. (80.0%), *Penicillium* spp. (40.0%) *Aspergillus* spp. (23.3%) and *Geotrichum* spp. (23.3%); Region of Capão Bonito, - *Fusarium* spp. (55.5%), *Penicillium* spp. (50.0%), *Geotrichum* spp. (38.9%) and *Aspergillus* spp. (22.2%); Region of Ribeirão Preto, - *Fusarium* spp. (77.8%), *Penicillium* spp. (50.0%) and Non Sporulated Fungi (5.5%) (Table 1).

The predominance of *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. in freshly harvested corn grains was also shown by Lillehoj and Zuber (20) in a work carried out with samples from different countries. Julian *et al.* (15) reported similar findings on corn from Honduras, as González *et al.* (11) in corn from five different regions of Argentina.

The *Fusarium* spp. frequencies, shown in Table 1, agree with data reported by other workers (7,17,24,27), whom describe this genus as the most prevalent filamentous fungus in freshly harvested Brazilian corn. At the species level, 60.6% were identified as *F. moniliforme* and 9.1% as *F. subglutinans*.

Among the *Aspergillus* isolates, the species identified were *A. flavus* (15.1%) and *A. glaucus* (3.0%). The presence of *A. flavus* in freshly harvested corn was previously observed (17,18,19). Although *Aspergillus* spp. has been typically considered a storage fungus, these findings demonstrate its field occurrence.

The high frequency of *F. moniliforme* (60.6%) and *A. flavus* (15.1%) in the samples surveyed in our work emphasizes the importance of research on fumonisin and aflatoxin contamination of freshly harvested corn. The presence and extent of fungal growth not only can indicate what mycotoxin is to be expected but also may point to adequate strategies for prevention of toxin production (23).

The results on CFU/g (Table 2) showed that *Fusarium* spp. prevailed in all the samples, with maximum values of 3.6×10^6 in Assis, 4.8×10^5 in Capão Bonito and 1.7×10^6 in Ribeirão Preto; these numbers stand above the tolerance limits (10^2 to 10^4 CFU/g) recommended by the International Commission on Microbiological Specification for Foods (13). The highest *Aspergillus* spp and *Fusarium* spp. counts were detected in samples from Assis; on the other hand, *Penicillium* spp. was detected as the main contaminant of corn grains from Capão Bonito.

The fungal isolates were recovered from corn with moisture content (MC) and water activity (a_w) at 5.0% to 20.2% and 0.30 to 0.99, respectively (Table 3). The highest frequencies of the three major toxigenic genera were detected in grains with a_w of 0.60 to 0.70 and MC 10% to 12%, which are values considered adequate for corn trading in Brazil (3).

Table 1: Absolute and relative frequencies (%) of fungi isolated from 66 samples of freshly harvested corn samples (hybrids BR-201, C-901 and CX-322) in Assis (30), Capão Bonito (18) and Ribeirão Preto (18) during February to December 1995 (State of São Paulo, Brazil).

Fungus	Planted Area					
	Assis		Capão Bonito		Rib. Preto	
	AF[a]	RF[b]	AF	RF	AF	RF
Fusarium spp.	24	80.0	10	55.5	14	77.8
Aspergillus spp.	7	23.3	4	22.2	0	0.0
Penicillium spp.	12	40.0	9	50.0	9	50.0
Geotrichum spp.	7	23.3	7	38.9	0	0.0
N.S.F.[c]	0	0.0	0	0.0	1	5.5

[a] Absolute Frequency;
[b] Relative Frequency;
[c] Non-sporulated fungi.

Table 2: Total counts of *Fusarium*, *Aspergillus* and *Penicillium* recovered from 66 samples of freshly harvested corn sampled in Assis, Capão Bonito and Ribeirão Preto (State of São Paulo, Brazil).

Fusarium spp.			
Region	CFU/g [a] x 10^4		
	BR-201	C-901	CX-322
Assis	400.0	418.0	1108.0
Capão Bonito	65.0	49.0	45.0
Ribeirão Preto	312.0	442.0	448.0
Aspergillus spp.			
Region	CFU/g x 10^4		
	BR-201	C-901	CX-322
Assis	86.0	200.0	19.0
Capão Bonito	7.0	6.0	4.0
Ribeirão Preto	ND[b]	ND[b]	ND[b]
Penicillium spp.			
Region	CFU/g x 10^4		
	BR-201	C-901	CX-322
Assis	64.0	24.0	9.0
Capão Bonito	13.0	83.0	37.0
Ribeirão Preto	14.0	15.0	14.0

[a.] CFU/g = Colony forming units per gram of food
[b] Not detected at dilution 10^4

Table 3 : Climatic data and moisture content and water activity of 66 samples of freshly harvested corn kernels (hybrids BR-201, C-901 e CX-322) collected in Assis, Capão Bonito and Ribeirão Preto (State of São Paulo, Brazil) from February to December 1995.

ASSIS REGION								
	Moisture content (%)			Water activity (a_w)			Rainfall	Temperature
	BR-201	C-901	CX-322	BR-201	C-901	CX-322	(mm)	(°C)
1	10.6	12.1	10.5	0.63	0.63	0.63	4.8	24.6
2	11.4	11.8	11.1	0.63	0.63	0.62	5.8	24.8
3	9.3	10.1	10.5	0.65	0.63	0.63	5.3	24.4
4	10.9	11.3	10.2	0.64	0.62	0.64	5.6	24.3
5	10.4	20.2	9.9	0.63	0.62	0.64	5.1	23.6
6	6.0	10.0	5.6	0.53	0.48	0.50	5.2	23.3
7	10.6	8.8	5.4	0.49	0.48	0.50	4.6	23.1
8	11.8	6.0	8.8	0.55	0.49	0.50	2.7	21.3
9	10.6	10.6	11.1	0.65	0.65	0.68	2.8	21.3
10	10.6	10.6	11.1	0.65	0.65	0.68	4.7	26.1
CAPÃO BONITO REGION								
	Moisture content (%)			Water activity (a_w)			Rainfall	Temperature
	BR-201	C-901	CX-322	BR-201	C-901	CX-322	(mm)	(°C)
1	11.0	13.5	14.0	0.68	0.69	0.70	4.7	22.1
3	10.5	11.6	10.8	0.61	0.62	0.30	5.5	22.2
5	11.3	11.8	10.6	0.74	0.73	0.99	5.0	20.9
7	5.0	16.8	16.2	0.60	0.54	0.53	3.0	19.5
9	8.6	8.9	9.3	0.57	0.54	0.56	3.1	18.9
11	11.8	11.3	10.6	0.61	0.60	0.62	2.7	19.0
RIBEIRÃO PRETO REGION								
	Moisture content (%)			Water activity (a_w)			Rainfall	Temperature
	BR-201	C-901	CX-322	BR-201	C-901	CX-322	(mm)	(°C)
1	11.5	12.0	12.5	0.69	0.73	0.74	6.3	25.1
3	10.6	9.6	10.8	0.65	0.65	0.65	7.4	24.7
5	9.6	11.4	9.4	0.65	0.65	0.66	6.3	23.9
7	10.6	11.0	10.3	0.61	0.61	0.62	5.2	23.0
9	9.0	8.6	9.0	0.56	0.52	0.53	6.3	24.2
11	9.1	12.2	9.4	0.59	0.58	0.58	6.3	24.2

The statistical data (Table 4) of abiotic factors indicated a significant influence of a_w on *Fusarium* spp. growth in hybrids BR-201 and CX-322 from the Assis region, and the models for these two hybrids differed significantly ($p<0.05$). In the Capão Bonito region, a strong correlation was detected between *Fusarium* spp. growth and rainfall for both BR-201 e CX-322, but the analysis of parallel lines indicated a similar behavior for these two hybrids. None of the independent variables analyzed affected the growth of *Fusarium* spp. in cultivar C-901 in both Assis and Capão Bonito, suggesting that, in this case, some other factor(s) must be exerting an effect on the degree of fungal contamination. In the Ribeirão Preto region, a significant correlation was shown between *Fusarium* spp. growth and a_w for hybrid BR-201 and between *Fusarium* spp growth and MT for both hybrids C-901 and CX-322.

An analysis of means per region showed that significant differences of *Fusarium* spp growth occurred in C-901 when comparing Capão Bonito with Ribeirão Preto ($p<0.05$). However, no significant regional differences were found for both BR-201 and CX-322 at the same level of significance.

The toxigenic potential of 10 *A. flavus* isolated from the 66 samples of freshly harvested corn grains (hybrids BR-201, C-901 e CX-322) was found to include 6 aflatoxin producers, with toxin concentrations ranging from 615 mg/kg to 30.750 mg/kg (AFB_1) and from 11 mg/kg to 22 mg/kg (AFB_2). Two (33.3%) of these strains produced only AFB_1 while 4 (66.7%) produced both AFB_1 and AFB_2.

Concerning the production of fumonisins by *F. moniliforme* isolates, the data showed that all the 40 strains tested were positive for FB_1 and FB_2; the concentration ranged from 20 mg/g to 2168 mg/g for FB_1 and 10 mg/g to 380 mg/g for FB_2. Out of the total analyzed, 4 strains produced only FB_1 (10.0%), 2 only FB_2 (5.0%) and 34 both FB_1 and FB_2 (85.0%).

The highest aflatoxin and fumonisin concentrations were detected among *A. flavus* and *F. moniliforme* isolates from the Assis region; these strains were recovered from hybrids C-901 and CX-322, respectively**.** Such regional high production of aflatoxins and fumonisins may reflect the effect of temperature on fungal growth and consequent mycotoxins production. Lacey *et al.* (16) showed that the ideal temperature concerning growth and mycotoxin production ranges from 22 to 28°C for *F. moniliforme* strains and 25 to 35°C for *A. flavus* strains; our temperature values recorded in the Assis region (21 to 26°C) fell within this range.

Although the detection of toxigenic fungi in a substrate does not necessarily indicate that mycotoxins are naturally occurring in the field, it alerts to the potential risk of contamination (4). If both the substrate and the environmental conditions are adequate for mycotoxin production, such risk may increase and reach dangerous proportions. Our data contribute to the understanding of regional factors that may influence the growth of toxigenic fungi in three different freshly harvested corn hybrids in Brazil.

RESUMO

Microbiota fúngica e produção de aflatoxinas e fumonisinas por cepas de fungos isoladas de híbridos de grãos de milho recém-colhidos

A microbiota fúngica de 66 amostras de três híbridos de grãos de milho recém-colhido, provenientes de 3 regiões do Estado de São Paulo – Brasil (Assis, Capão Bonito e Ribeirão Preto), foram analisadas perante a influência dos fatores

Table 4: Statistical model, p-value and significant variable selected for hybrids BR-201, C-901 and CX-322, collected in Assis, Capão Bonito and Ribeirão Preto (State of São Paulo, Brazil).

ASSIS				
Hybrid	Model	p	R^2 (%)	Significant Variable
BR-201	$Fus= 48096 + 258405\ a_w - 459185\ a_w^2 + 270154\ a_w^3$	P<0.0021	90.00	a_w
C-901	*	*	*	*
CX-322	$Fus= -189651 + 980603\ a_w - 1677289\ a_w^2 + 9502116\ a_w^3$	P<0.0001	98.77	a_w
CAPÃO BONITO				
Hybrid	Model	p	R^2 (%)	Significant Variable
BR-201	$\sqrt{Fus+1}= 34982 - 18867R + 2507R2$	P<0.0069	96.37	R
C-901	*	*	*	*
CX-322	$\sqrt{Fus+1}= 273616 - 122647R + 14125R2$	P<0.0556	85.43	R
RIBEIRÃO PRETO				
Hybrid	Model	p	R^2 (%)	Significant Variable
BR-201	$\sqrt{Fus+1}= 14827 - 72008\ a_w + 116263\ a_w^2 - 62384\ a_w^3$	P<0.0295	98.02	a_w
C-901	$\sqrt{Fus+1}= 1559588 - 6143MT$	P<0.0133	81.79	MT
CX-322	$\sqrt{Fus+1}= 1373227 - 53711\ MT$	P<0.0671	60.88	MT

*= No model available; a_w = water activity; R= rainfall ; MT=mean temperature; P =p-value ; R^2= correlation coefficient

abióticos (teor de umidade, atividade de água, precipitação pluvial e temperatura média) na frequência de isolamento de fungos, bem como a potencialidade toxigênica das cepas de *Aspergillus flavus* e *Fusarium moniliforme* quanto à produção de aflatoxinas e fumonisinas, respectivamente. As análises microbiológicas demonstraram predominância de *Fusarium* spp., *Penicillium* spp. e *Aspergillus* spp. e outros dois gêneros de fungos filamentosos, isolados de grãos com atividade de água entre 0,30 e 0,99 e teor de umidade entre 5,0% e 20,2%. Entre *Fusarium* spp, *F.moniliforme* foi a mais frequentemente isolada, enquanto que, em relação ao gênero *Aspergillus*, predominou *A. flavus* nas três regiões. Todas as cepas de *Fusarium moniliforme* isoladas (40), produziram fumonisinas, que variaram de 20 mg/g a 2168 mg/g (FB_1) e 10 mg/g a 380 mg/g (FB_2). Referente a 10 cepas de *Aspergillus flavus* isoladas, 6 cepas (60,0%) produziram aflatoxinas, que variaram de 615 mg/kg a 30.750 mg/kg (AFB_1) e 11 mg/kg a 22 mg/kg (AFB_2).

Palavras-chave: Aflatoxinas, *Aspergillus flavus*, fumonisinas, *Fusarium moniliforme,* milho

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